Gene-Specific Methylation and Subsequent Risk of Colorectal Adenomas among Participants of the Polyp Prevention Trial

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Abstract

Hypermethylation of tumor suppressor and other regulatory genes is thought to play an important role in colorectal neoplasia and tumorigenesis. This study examined the association between gene methylation status in baseline adenomas and subsequent adenoma recurrence in a randomized dietary intervention study, the Polyp Prevention Trial. The methylation status of four genes [*CDKN2A* (*p16*), *PTGS2* (*COX2*), *ESR1* (*ER-α*), and *PGR*(*PR*)] was determined by MethyLight in 284 baseline adenomas from 196 trial participants. The association of gene methylation with recurrence was determined using logistic regression models. Gene methylation was evaluated as percent of methylated reference, a measure of methylation of each gene relative to control DNA. *ESR1*methylation status was inversely associated with adenoma recurrence, odds ratio = 0.36 (95% confidence interval, 0.15-0.88; P = 0.02) for the highest compared with the lowest quartile of the *ESR1*methylation. Further, *ESR1* methylation status was inversely associated with the recurrence of multiple adenomas, advanced adenomas, and the recurrence of adenomas in the proximal but not distal bowel. No association between *CDKN2A*, *PTGS2*, or *PGR* methylation and adenoma recurrence was observed. These data suggest that *ESR1* methylation may play a role in subsequent adenoma recurrence. (Cancer Epidemiol Biomarkers Prev 2005;14(5):1219–23)

Introduction

Colorectal cancer is the third leading cause of cancer-related deaths worldwide. A majority of colorectal cancers are thought to arise through a series of steps, which include initial hyperproliferation of normal epithelial cells, formation of aberrant crypt foci and adenomas, and finally the transition to invasive carcinomas (1, 2).

In addition to genetic abnormalities, another potential mechanism that may be important in the progression of colorectal cancer is promoter DNA hypermethylation. Aberrant methylation of the CpG islands in the promoters of genes that are normally unmethylated has been linked to transcriptional silencing of that gene. A growing number of tumor suppressor genes and other regulatory genes have been shown to be hypermethylated in colorectal cancer. Prior studies have shown that colorectal cancers and adenomas (about 15-50%) have what is termed to be a ''CpG island methylator phenotype,'' a condition characterized by the concurrent hypermethylation of a number of cancer-specific genes (3-7). In addition to promoter region hypermethylation, colorectal tumors have also been shown to possess widespread hypomethylation of CpG dinucleotides in noncoding parts of the genome, including repetitive elements (8). Thus, a global phenomenon of DNA methylation dysregulation may be occurring and contributing to the genesis and progression of colorectal cancers.

The causes of gene methylation aberrancies and their prognostic significance have not been established. Evidence suggests that individuals may be prone to methylation aberrancies as a consequence of genetic, dietary, or environmental exposures. Based on their study findings, Ricciardiello et al. (9) postulate that hypermethylation of the *MLH1* gene (resulting in the loss of *MLH1* expression) accounts for about 25% of adenomas that developed in subjects with a family history of colorectal cancer. The prevalence of *MLH1* methylation was significantly higher in adenomas of those patients with a family history of colorectal cancer versus those without. Further, *CDKN2A* methylation in colorectal tumors has been associated with age, gender, and anatomic location (10, 11). For example, *CDKN2A* methylation was six times more likely to occur in colorectal tumors in women compared in men, and nine times more likely to occur in tumors located in the proximal colon compared with those in the distal bowel.

In this study, we evaluated the association of the methylation status of four genes with subsequent adenoma recurrence among participants of a randomized, dietary intervention trial, the Polyp Prevention Trial. The genes were selected, in part, based on data derived from studies suggesting that there are two distinct types of promoter gene methylation in colorectal cancer, one involving gene methylation in normal colon as a function of age (type A, i.e., *ESR1*) and the other involving genes exclusively methylated in neoplasia (type C, i.e., *CDKN2A*; ref. 3, 12). We included the *PGR* gene because it is part of the estrogen signaling pathway and shown to be methylated in other cancers (13, 14). We were interested in whether the presence of gene methylation in adenomas removed at the baseline colonoscopy visit was associated with adenoma recurrence detected at subsequent visits.

Subjects and Methods

Subjects and Specimens. The participants of the study are a subgroup of enrollees in the Polyp Prevention Trial, a

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randomized, dietary intervention trial designed to test whether a low-fat, high-fiber, fruit and vegetable diet can inhibit the recurrence of colorectal adenomas. The Polyp Prevention Trial was a collaboration between eight study centers and the National Cancer Institute and was approved by the Institutional Review Boards of all participating centers. The overall design, rationale, and trial results have been previously reported (15, 16). The trial findings showed that the rate of recurrent adenomas was not changed by the dietary intervention (15). This study consisted of 196 patients selected from those members of the study population (both intervention and control groups) who had completed the trial, had adequate polyp tissue slides available to provide sufficient DNA for methylation analysis, and included enough women participants to examine the influence of hormone replacement therapy on *ESR1* methylation status. This sampling scheme resulted in a study population consisting of (*a*) women taking hormone replacement therapy at the baseline visit ($n = 58$); (*b*) women never taking hormones (at any visit; $n = 59$); and (*c*) men ($n = 79$). The three groups were frequency matched by age (± 3 years). The polyp recurrence rate among the 196 subjects was not significantly different from that of the overall trial population ($P = 0.20$).

Methylation Analyses of Polyp Tissue. Polyp tissue was dissected from two 10-μm tissue sections by scraping the cells with a 25-gauge needle. Cells were lysed in a solution containing 100 mmol/L Tris-HCl (pH 8.0), 10 mmol/L EDTA (pH 8.0) 1 mg/mL proteinase K, and 0.05 mg/mL tRNA at 50°C overnight. The DNA from the cell lysate was bisulfite converted as previously described (17) and then purified using the a QIAamp 96 Blood Kit (Qiagen, Valencia, CA) according to the specifications of the suppliers. After purification, the bisulfite-converted DNA was desulfonated in 0.076 mol/L NaOH for 15 minutes at room temperature and then neutralized with HCl. Then a second purification step was done using the same QIAamp 96 Blood Kit to obtain bisulfite-converted DNA of high quality suitable for MethyLight analysis.

DNA methylation analysis was done by MethyLight as previously described (17, 18). All reactions used probes containing a 6-FAM fluorophore at the 5′ end and a black hole quencher (BHQ) at the 3′ end. Methylation reactions were done for *CDKN2A*-M2, *PTGS2*-M1, and *ESR1*-M1 using previously described primer and probe sequences (18). The *PGR*-M1 primer and probe sequences (in the 5′ to 3′ direction) are as follows: forward primer, GGCGGTGACGGTCGTATTC; reverse primer, ACAAACCGTCCCGCGAA; and probe, 6-FAM-AACAACCGCTCGCGCCCGA-BHQ. Control reactions for *ACTB* and *COL2A1* were used to normalize for the amount of input DNA in each reaction. *Sss*I-treated human peripheral blood leukocyte DNA was used as a fully methylated reference (18). The percentage of methylated reference (PMR) was calculated as previously described (18). Briefly, the PMR of specific genes was calculated by dividing the gene of interest/ACTB ratio of each sample by the ratio of a control sample (*Sss*I-treated leukocyte DNA) and multiplying by 100. This was repeated for the second control gene, *COL2A1*, and the two values were averaged to obtain the PMR.

Statistical Analyses. All statistical analyses were conducted using STATA 8.0 (College Station, TX). The main analyses examined the association between gene methylation in baseline adenomas and adenoma recurrence over the course of the trial (between the T1 and T4 visits). Gene methylation was evaluated as percent methylation relative to a methylated reference DNA (PMR). PMR values were ranked and categorized into quartiles or tertiles. In the case where there was methylation data for more than one polyp per person, a single measure for methylation status per person was generated by averaging the PMR values across all polyps for each of the genes. The average PMR values for each of the genes were then ranked and categorized into quartiles or tertiles for subsequent analyses. To evaluate methylation status across all of the genes, we created a methylation index by adding PMRs for each gene and categorizing this composite into quartiles based on rank. The Wilcoxon rank-sum test was used to test for differences in recurrence status for continuous variables including PMR, age, and body mass index. The χ^2 test was used for categorical variables. The association between gene methylation and recurrence was computed using logistic regression. Potential confounders were evaluated by assessing their association both with adenoma recurrence and gene methylation status. The association between gene methylation and adenoma multiplicity was evaluated using logistic regression with the outcome variable being those with more than one recurrent adenoma compared with those with a single adenoma or no adenoma recurrence as the reference group. The association between gene methylation and recurrence of advanced adenomas was evaluated using logistical regression with the outcome variable being those with recurrence of an advanced adenoma compared with those with recurrence of non-advanced adenomas or no recurrence as the reference group. An adenoma(s) was considered to be ''advanced'' if it had a maximal diameter of at least 1 cm, had at least 25% villous elements, or displayed evidence of high-grade dysplasia. Logistic regression was also used to estimate the odds ratio and 95% confidence interval (OR; 95% CI) for recurrence of adenomas for each anatomic subsite (i.e., proximal, distal, or both, a combination of distal and proximal) relative to the no adenoma reference group (those with recurrent adenomas at locations other than the subsite being evaluated were not included in the analysis).

Results

This study included an analysis of the methylation status of four genes (*CDKN2A*, *PTGS2*, *ESR1*, and *PGR*) in baseline adenomatous polyps from a selected group of participants of the Polyp Prevention Trial. The methylation status of the genes was evaluated by MethyLight analysis in 284 polyps from 196 patients (multiple polyps were evaluated in 60 of the patients). A description of selected baseline characteristics related to colorectal cancer risk by recurrence group is presented in Table 1. A higher proportion of men had an adenoma(s) recurrence; however, there were no significant differences for any of the other study factors, including age, smoking, alcohol consumption, hormone replacement therapy or nonsteriodal anti-inflammatory drug use, and family history of colorectal cancer, between participants who had or did not have an adenoma recurrence.

The percentage of adenomas having any detectable gene methylation (PMR > 0) and the median and interquartile range of PMR values for the four genes according to recurrence status group are presented in Table 2. The prevalence of gene methylation in adenomas was high for *CDKN2A*, *PTGS2*, and *ESR1* (all being above 80%); however, the range of PMR values varied substantially. The median PMR values among all the participants' adenomas were 16, 7, and 122 for *CDKN2A*, *PTGS2*, and *ESR1* methylation, respectively. *ESR1* methylation showed statistically significant differences in the median levels of PMR between recurrence status, with higher *ESR1* methylation observed in adenomas of patients that did not have a recurrence ($P < 0.01$). PMR values observed for *CDKN2A*, *PTGS2*, and *PGR* did not differ by recurrence group.

The association of gene methylation with adenoma recurrence is presented in Table 3. The patients were ranked and classified into PMR quartiles for each of the genes. In the case of those patients where there were methylation data available

Table 1. Selected baseline characteristics according to adenoma recurrence status among nested study participants of the Polyp Prevention Trial

	Overall (n = 196)	Any polyp recurrence (n = 69)	No polyp recurrence (n = 127)	P*
Age, mean years (SD)	59.9 (8.3)	61.0 (8.4)	59.3 (8.3)	0.18
Body mass index, mean (SD)	27.4 (3.9)	27.6 (4.0)	27.2 (3.9)	0.56
Gender, % male	40.3	50.7	34.7	0.03
Current smoker, %	23.0	29.0	20.3	0.34
Current alcohol use, %	55.1	52.2	56.7	0.54
Current hormone replacement therapy use, % (women only)	49.6	41.2	53.0	0.24
Current nonsteriodal anti-inflammatory drugs use, %	11.2	7.3	13.4	0.19
Family history of colorectal cancer, %†	31.6	34.8	29.9	0.49

NOTE: Adenoma recurrence is defined as any adenomatous polyp recurrence diagnosed between T1 colonoscopy and T4 colonoscopy.
*P values for differences between recurrence status in means determined by t test and differences in proportions determined by χ^2 test.
†Family history is defined as having a first-degree relative with colorectal cancer.

for more than one polyp, the average PMR value across all available polyps was used. The association of methylation status of *CDKN2A*, *PTGS2*, and *ESR1* with adenoma recurrence was determined using logistic regression adjusting for sex and polyp multiplicity (these were identified as predictors of adenoma recurrence in the original analyses of the Polyp Prevention Trial findings, and thus were included in the final model). The association with *PGR* was not evaluated due to its low prevalence of methylation. Methylation of *ESR1* was inversely associated with adenoma recurrence (OR = 0.36; 95% CI, 0.15-0.88, for the highest compared with the lowest quartile of methylation). Methylation of *CDKN2A* and *PTGS2* was not associated with adenoma recurrence. Although *ESR1* methylation was associated with the size of the polyp (with the larger polyps having more extensive methylation; $P < 0.01$), polyp size was not associated with recurrence, and adjustment by polyp size did not affect the association between *ESR1* methylation and adenoma recurrence. Because detection of gene methylation might depend on the number of adenomas evaluated, an indicator variable for number of polyps was added to the regression models and we did a subgroup analysis of subjects with only one polyp. The risk estimates were not materially altered by either approach.

ESR1 methylation status in baseline adenomas did not differ by gender nor did it differ according to hormone replacement therapy status among women (data not shown).

The association between *ESR1* methylation and recurrent adenoma characteristics, such as advanced polyp, multiple polyp, and polyp location in the large bowel, was evaluated (Table 4). *ESR1* methylation showed a strong inverse association with both recurrence of advanced adenoma(s) and recurrence of multiple adenomas (i.e., two or more adenomas). Interestingly, *ESR1* methylation status was also associated with the recurrence of proximally located adenomas but not with recurrence of adenomas in the distal bowel. Because *CDKN2A* methylation status has been reported to occur at a higher frequency in proximal tumors and in tumors from women, we also evaluated *CDKN2A* methylation across gender and location and found no differences (data not shown).

Table 2. Comparison of gene methylation levels in baseline adenomas according to recurrence status among a subset of participants of the Polyp Prevention Trial

	Recurrence (n = 69)		No recurrence (n = 127)		P*
	% positive	PMR† median (IR)‡	% positive	PMR† median (IR)‡	
CDKN2	81.0	15 (3-43)	72.4	17 (5-50)	0.40
PTGS2	91.3	7 (4-17)	89.0	6 (3-19)	0.70
ESR1	88.4	101 (39-162)	88.2	143 (79-238)	0.006
PGR	13.4	4 (3-44)	12.7	6 (2-44)	0.84

*P value refers to the comparison of the ranks of the median scores and was computed by Wilcoxon rank-sum test.
†Gene methylation evaluated as PMR. Averaged PMR scores across polyps were used when methylation data were available for more than one polyp per patient.
‡Median and interquartile range (IR) for polyps with at least some methylation only were included in the analysis.

Discussion

This study describes an analysis of the association between gene methylation of four genes in the baseline adenomas and adenoma recurrence among a subgroup of participants of a randomized intervention trial. We hypothesized that gene hypermethylation in the adenoma(s) taken at the baseline colonoscopy visit could serve as a biomarker of increased risk for subsequent adenoma recurrence. In contrast, we found a significant reduction in risk of recurrence among participants with the highest level of *ESR1* methylation in their baseline adenomas. Methylation of *CDKN2A* and *PTGS2* or an overall gene methylation index was not associated with adenoma recurrence. The prevalence of *PGR* methylation in adenomas was too low to statistically evaluate an association with recurrence.

Our hypothesis was based, in part, on the postulated existence of a ''methylator phenotype'' in a subset of colorectal cancers, particularly those in the proximal bowel. Toyota et al. (3) hypothesized that CpG methylation of genes falls into two categories: type A, age related, where the gene is methylated in normal colon mucosa and the degree of methylation increases with age; and type C, cancer related, where methylation is specific to neoplastic or preneoplastic tissue. The genes selected in our analysis included representatives of both categories: *ESR1*, a type A gene, versus *CDKN2A*, a type C gene.

We did not find evidence for the correlation of methylation of any of the four genes with age, anatomic location of the adenomas, or gender. In contrast, Wiencke et al. (10) observed a 6-fold increase in *CDKN2A* methylation in colorectal tumors among women compared with men independent of age, degree of differentiation, and anatomic location. In addition, proximal tumors were nine times more likely to have *CDKN2A* methylation than tumors located distally. In another study, Hawkins et al. (19) found associations between *CDKN2A* methylation and age, sex, and anatomic location, with gene methylation occurring more frequently in elderly women and among proximal tumors. In contrast, Xiong et al. (11) evaluated the methylation status of four genes (including *CDKN2A* and *ESR1*) in microsatellite instability (+) and microsatellite instability (−) colorectal tumors and found no association between the methylation status of any of the genes

Table 3. The association of gene-specific methylation in baseline adenoma(s) and adenoma recurrence among a subset of participants of the Polyp Prevention Trial

Gene	Ranked PMR quartiles*			
	Q1	Q2	Q3	Q4
	OR (95% CI)†, # recur/# no recur	OR (95% CI), # recur/# no recur	OR (95% CI), # recur/# no recur	OR (95% CI), # recur/# no recur
CDKN2	1.00 (ref) 12/34	1.77 (0.73-4.30) 19/30	1.59 (0.64-3.94) 17/28	1.26 (0.50-3.14) 15/31
PTGS2	1.00 (ref) 19/42	1.25 (0.54-2.94) 16/25	1.01 (0.44-2.35) 16/29	1.09 (0.48-2.47) 18/31
ESR1‡	1.00 (ref) 23/25	0.52 (0.23-1.21) 17/32	0.59 (0.26-1.36) 18/31	0.36 (0.15-0.88) 11/39
MI§	1.00 (ref) 18/22	0.53 (0.23-1.24) 19/42	0.59 (0.24-1.45) 14/31	0.62 (0.24-1.49) 16/31

*Gene methylation evaluated as PMR categorized into quartiles with Q1 being the lowest and Q4 the highest rank of PMR. Averaged PMR scores across polyps were used when methylation data were available for more than one polyp per patient.
†OR and 95% CIs adjusted for age, gender, and multiple polyps at baseline (yes/no).
‡P value for trend based on continuous variable, $P = 0.02$.
§Methylation index (MI) generated by adding PMRs for each gene and categorizing into quartiles based on rank. Averaged PMR score used for participants with methylation data available for more than one polyp.

and anatomic subsite, gender, or microsatellite instability status. Only *ESR1* methylation was associated with age, with a higher extent of methylation observed in tumors from older women.

CDKN2A is a tumor suppressor gene and its expression is reduced in colorectal tumors. The presence of *CDKN2A* methylation in both adenomas and colorectal cancer has been well documented (3, 4, 10, 11). For example, Toyota et al. (4) evaluated *CDKN2A* methylation in 45 adenomas and 88 colorectal cancers and found *CDKN2A* methylation in 50% of adenomas and in 53% of colorectal cancers.

Although it is generally thought that up-regulation of *PTGS2* expression is involved in colorectal carcinogenesis, a subset of tumors have shown a loss of *PTGS2* expression, coincident with hypermethylation. In one study, *PTGS2* methylation was observed in 13% of sporadic colorectal cancers and in 14% of colorectal adenomas (20).

Our finding of a high prevalence of extensive *ESR1* methylation in adenomas is supported by prior studies (11, 12). Although *ESR1* methylation is present in normal colonic tissue, colorectal tumors and adenomas show more extensive *ESR1* methylation. Although we observed some methylation of *ESR1* in 88% of all the adenomas, the extent of methylation across the adenomas was highly variable (the PMR ranged from 1% to 700%). We found an association between *ESR1* methylation and the recurrence of proximally located adenomas but no association with the recurrence of adenomas in the distal bowel. In a prior study, *ESR1* methylation was found to be highest in the ''normal'' colonic tissue from the proximal bowel, suggesting a potential field effect for *ESR1* methylation in the proximal bowel (12).

Our observation of reduced risk of adenoma recurrence with *ESR1* methylation seems to be in conflict with current theories of promoter-region gene methylation and tumorigenesis. According to this theory, extensive methylation of *ESR1* would result in adenomas with reduced ER protein expression. There are two major forms of *ER*, *ESR1* (*ER-*α) and *ESR2* (*ER-*β). *ESR1* has been shown to be present in low levels in the colon with no differences in mRNA or protein expression between normal colon, adenomas, and colon cancer (21, 22). *ESR2* is the predominant form in normal colon and its expression is shown to be reduced in colorectal cancer. Weyant et al. (23) showed that relative levels of *ESR1* and *ESR2* expression were associated with inhibition of tumor formation in the Min/+ mouse model. Administration of 17β-estradiol inhibited the number of tumors formed and this inhibition was concomitant with a decrease in *ESR1* and an increase in *ESR2* expression in the colon. Thus, a possible explanation of our findings is that a more extensive *ESR1* methylation, corresponding to reduced protein expression, is beneficial for inhibition of tumor formation in the colon.

It is possible that the reduced risk for recurrence associated with extensive *ESR1* methylation may not be a result of alterations in *ER* itself. *ESR1* methylation may be a marker for some other molecular or cellular process related to recurrence. For example, methylation of *ESR1*, a type A gene, may reflect cell proliferation, senescence, or the rate of development of the polyp.

Table 4. The association of *ESR1* gene methylation in baseline adenoma(s) with recurrent adenoma characteristics and anatomic location among a subset of participants of the Polyp Prevention Trial

Adenoma characteristic	OR (95% CI)* (# recurrent/# no recurrence)			P trend†
	ESR1 methylation tertile			
	T1	T2	T3	
Advanced recurrence vs no advanced recurrence	1.00 (ref) 6/50	0.52 (0.14-1.95) 4/64	0.24 (0.05-1.23) 2/70	0.08
Multiple recurrence vs no multiple recurrence	1.00 (ref) 13/43	0.57 (0.23-1.42) 10/58	0.25 (0.09-0.74) 5/67	0.05
Anatomic subsite vs no adenoma recurrence				
Proximal recurrence	1.00 (ref) 11/30	0.57 (0.21-1.55) 10/44	0.25 (0.07-0.81) 4/53	0.03
Distal	1.00 (ref) 10/30	0.60 (0.22-1.67) 9/44	0.64 (0.24-1.71) 11/53	0.39
Both	1.00 (ref) 5/30	0.58 (0.14-2.37) 5/44	0.62 (0.14-2.69) 4/53	0.52

NOTE: *ESR1* methylation evaluated as PMR categorized into tertiles with T1 being the lowest and T3 the highest rank of PMR. Averaged PMR scores across polyps were used when methylation data were available for more than one polyp per patient. Advanced adenoma defined as one that had a maximal diameter of at least 1 cm or at least 25% villous elements or evidence of high-grade dysplasia (including carcinoma). Proximal is defined as the portion of the large bowel from the cecum up to, but not including, the splenic flexure. Distal is defined as the portion of the large bowel from the splenic flexure and including the rectum. Both are defined as having proximal and distal adenoma recurrence.
*OR and 95% CIs adjusted for age, gender, and multiple polyps at baseline (yes/no).
†P value for trend based on continuous PMR variable.

This study is strengthened by its conduct within a prospective clinical trial cohort that allowed for a detailed, controlled follow-up designed for the complete detection of adenoma recurrence. In addition, the evaluation of gene methylation in multiple adenomas per patient potentially allows for the improved classification of methylation status. Finally, the MethyLight technique used in this study allowed for quantitation of gene methylation, and the extent of gene methylation might be more biologically relevant than a categorical assessment of gene methylation in the study of adenoma recurrence.

In conclusion, this prospective study conducted within a clinical recurrence trial suggests that *ESR1* gene methylation status in adenomas may have prognostic significance. This may have clinical relevance to colorectal cancer screening practice because individuals with recurrent adenomas are thought to be at increased risk for the development of colorectal cancer. The presence of low *ESR1* methylation may indicate a need for increased surveillance or more frequent colonoscopy. These findings are intriguing but need to be confirmed in other study populations.

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